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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Achenbach		
Serial No.	:	09/463,851	Examiner:	Patten, P.
Filed	:	June 5, 2000	Group Art Unit:	1651
For	:	PHARMACEUTICAL COMPOUNDS ISOLATED FROM ARISTOLOCHIA TALISCANA		

DECLARATION OF PETER HYLANDS
UNDER 37 C.F.R. § 1.132

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

March 10, 2003
Date of Deposit

Carmella L. Stephens
Attorney Name

41,328
Registration No.

Carmella L. Stephens
Signature

March 10, 2003
Date of Signature

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Peter Hylands, residing at Laundry Cottage, Yewleigh Lane, Upton upon Severn, WR8 0QW, United Kingdom, do declare:

1. I currently hold the position of Research Director at Oxford Natural Products plc where I have been employed since April 1998. The research data included in this declaration

NY02:423983.2

was obtained in a barter swap between Oxford Natural Products plc and Proteome Sciences plc, the parent company of the assignee of this application Proteome Sciences Inc, whereby Oxford Natural Products plc carried out work for Proteome Sciences plc during which the data included in this declaration was obtained, in exchange for work carried out by Proteome Sciences plc for Oxford Natural Products plc in the field of proteomes. Experiments described herein were performed under my supervision as Chief Scientific Officer of Oxford Natural Products plc at Department of Pharmacy, King's College London. A copy of my Curriculum Vitae is attached herewith as Exhibit A.

2. I have read the above-identified application and it is my understanding that the invention disclosed in the above-identified application relates to extracts isolated from *Aristolochia taliscana*, methods of using the extracts, compounds isolated from the extracts and methods of using the compounds. The extracts are useful as agents to inhibit mutagenesis, fungal growth, and inflammation.

3. Inflammation is the local accumulation of fluid, plasma proteins, and white blood cells that may result from physical injury, infection, or a local immune response. While acute inflammation relates to early and transient conditions, chronic inflammation is associated with persistent infections, and chronic diseases, such as inflammatory bowel disease, rheumatoid arthritis, synovitis, and psoriasis.

4. Stimulation of the inflammmatory response results in arachidonic acid metabolism generated via the lipoxygenase (LOX) and cyclooxygenase (COX) pathways. Stimulation of the LOX pathway can be detected by measuring the formation of leukotrienes, such as leukotriene B_4 (LTB_4), which exhibit proinflammatory activity. Stimulation of the COX pathway can be

detected by measuring prostaglandins, such as prostanglandin E_2 (PGE_2), and thromboxane B2 (TXB_4), which also induce inflammation.

5. Experiments were conducted to evaluate the effectiveness of extracts isolated from *Aristolochia taliscana* and compounds found therein on the inflammatory response. Specifically, the ability of eupomatenoid compounds derived from *Aristolochia taliscana* extracts were tested for their ability to inhibit the production of LTB_4, PGE_2 and TXB_4.

6. Dried samples of *A. taliscana* leaves were first extracted by maceration for 4 x 48 hours in 3-5 volumes of petroleum ether. Successive extracts were decanted after each 48-hour period, evaporated to dryness and combined. Exhibit B shows the scheme for the identification and isolation of eupomatenoid-1, eupomatenoid-7, eupomatenoid-8 and licarin A by successive chromatographic separations from *A. taliscana* extracts. Initially, the separations were characterized, and their fidelity confirmed using small batches of dried *A. taliscana* approximately 20g. The isolation of eupomatenoid-7 batch sizes of 1-1.5kg were required during scale up procedures.

7. Fractions containing single compounds were evaporated to dryness and the compound re-crystallized from hexane. All columns were packed with chromatographic grade silica, and had dimensions appropriate to the scale of the quantity of crude extract. Elution under gravity was performed with the following solvent systems. (volumes appropriate to scale):

<u>Column 1</u>:light petroleum (br 40°-60°)/$CHCl_3$ 70/30, 65/35/ 50/50. 30/70; $CHCl_3$ 100%; $CHCl_3/CH_3OH$ 80/20, 50/50. <u>Column 2</u>: light petroleum/$CHCl_3$ 80/20, 70/30, 50/50. <u>Column 3</u>:

light petroleum/$CHCl_3$ 70/30, 50/50. Column 4: Hexane/$CHCl_3$ 2/1. Column 5: Hexane/$CHCl_3$ 60/40. Column 6: Hexane/$CHCl_3$ 60/40. Column 7: Hexane/$CHCl_3$ 2/1.

8. Eupomatenoids-1 and -7 (E1, E7) were analyzed by Time-of-Flight Mass Spectroscopy (TOF-MS) and proton Nuclear Magnetic Resonance Spectroscopy (NMR) and compared to pure compounds to confirm their identities. The authentic compounds were used as reference standards to guide the isolation of those two constituents from the extract using thin-layer chromatography (mobile phase chloroform:hexane 8:3 v/v; development with anisaldehyde reagent and visualization under UV light at 254nm). Purification of sufficient quantities of E7 required scaling up of the extraction/isolation process, and combining final chromatographic fractions.

9. The identities of eupomatenoid-8 (E8) and licarin A were also confirmed by rigorous analysis by TOF-MS and NMR, before the compounds were submitted for biological testing.

10. Exhibit C shows the chemical structures of the isolated eupomatenoid compounds.

11. The effects of the isolated eupomatenoid compounds on inflammation were determined by measuring the generation of prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) for the divergent COX pathway, or leukotriene B_4 (LTB_4) for the LOX pathway using an *in vitro* rat leukocyte cell model.

12. Mixed leukocytes were obtained from female Wistar rats treated 16 hours previously with 10ml 6% oyster glycogen by peritoneal lavage. The leukocytes were resuspended at 2.5 x 10^6 cells/ml in Hanks Buffered Saline Solution at 37°C. The cells were then incubated for 5 minutes with each test compound (added in 2μl of DMSO, at final concentrations of 5, 10, 25 or

$50\mu g/ml$, n=3) prior to challenge with the calcium ionophore, A23187, for 10 min. The cells were pelleted down and the levels of PGE_2, TXB_2 and LTB_4 were measured in the supernatant.

13. The addition of the calcium ionophore A23187 to the mixed leukocyte suspensions produced a robust response, with PGE_2 formation stimulated approximately 6-fold (Exhibit D), TBX_2 generation stimulated 30-fold (Exhibit E), and LTB_4 generation stimulated 40-fold (Exhibit F).

14. Exhibit E shows that E1 inhibited the generation of TXB_2 by approximately 80% at concentration of 5, 10, 25 or $50\mu g/ml$ (Exhibit E). The formation of PGE_2 was also inhibited, although the effect was not as consistent (Exhibit D). LTB_4 generation did not appear to be inhibited by E1 (Exhibit F). The results with E1 demonstrate that E1 is capable of inhibiting the COX pathway and less effective at inhibiting the LOX pathway.

15. E7 produced a profound inhibition of TXB_2 formation (Exhibit E) and similarly reduced the generation of PGE_2 (Exhibit D). The effect was not restricted to the COX pathway, as a strong inhibitory effect was also seen against LTB_4 generation (Exhibit F).

16. When tested, E8 did not appear to inhibit TXB_2 generation (Exhibit E) and had little or no effect on the formation of PGE_2 (Exhibit D) or LTB_4 (Exhibit F), except at the highest concentration.

17. Like E7, licarin A had a general effect to inhibit A23187-initiated stimulation of TXB_2 and TB_4, and a reduced generation of PGE_2 (Exhibit D, E, F).

18. Any potential for cytotoxicity was assessed by measurement of changes in the activities of myeloperoxidase (MPO) and lactate dehydrogenase (LDH) enzymes.

19. Supernatants from A23187-treated cells were tested for MPO enzyme activity. Such enzyme activity was detectable by measuring the conversion of the substrate hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCl). Total cellular content of MPO was measured after lysis by the detergent Triton X-100.

20. Cytotoxicity was also assessed by measurement of LDH activity in the supernatant following a fifteen-minute pretreatment with the test compounds. Enzyme activity was measured indirectly by the specific absorption at 340nm of reduced NADH. Triton X-100 was used to solubilize cells, to provide a measurement of total cellular LDH content.

21. The appearance of MPO activity in the medium was increased almost 8-fold above the basal levels in unstimulated cells within 5 minutes of exposure to A23187, and more than 10-fold by ten minutes. Exhibit G and H show that the addition of Triton X-100 produced a maximal release of approximately 18-fold within 5 minutes. Exhibit I shows that the release of LDH was increased more than 4-fold by the addition of A23187 and approached 8-fold when Triton X-100 was added.

22. The MPO release was unaffected by E1 at 50µg/ml (Exhibit G), although there was a slight elevation of the level of LDH at this concentration (Exhibit I). Thus, E1 has little or no cytotoxic effects on the cells.

23. Using the MPO assay, E7 at 50μg/ml produced a stimulation of MPO release beyond that obtained by A23187, almost to the extent achieved by cell lysis with the detergent (Exhibit G), suggesting a powerful cytotoxic effect. A similar response seen at 10 and 25μg/ml (Exhibit H). The E7 cytotoxic effect was also observed in the LDH assay at all concentrations above 5μg/ml (Exhibit I).

24. Using the MPO assay, E8 appeared to abolish MPO release and/or activity at 10, 25 and 50 μg/ml (Exhibit G and H). The mechanism responsible for the inhibition of MPO release and/or activity is not clear, but may reflect a general cytoprotective property of the compound since there was also an small inhibition of LDH release (Exhibit I). Alternatively, E8 may be able to scavenge the substrate hydrogen peroxide, or inhibit the MPO enzyme.

25. MPO release was increased by licarin A (Exhibit G) and reflected in the measurements of LDH release (Exhibit I). Thus, licarin A exhibits a strong cytotoxic effect.

26. These results demonstrate that E1 is a selective inhibitor of the COX pathway, with little or no propensity for cytotoxicity and thus acts as a potent inhibitor of the inflammatory response. Although E8 appears to be a weak inhibitor of LTB_4, PGE_2 and TXB_4 production, the compound does seem to have some cytoprotective properties. Although E7 and licarin A exhibit some cytotoxic effects, these compounds are capable of inhibiting the formation of the LTB_4, PGE_2 and TXB_4 and have potential for use as inhibitors of the inflammatory response. Table 1 summarizes the results of the experiments presented herein.

27. I hereby declare that all statements made herein by my own knowledge are true, and that all statements made on information and belief are believed to be true, and further that I make these statements with the knowledge that willful false statements, and the like, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patent issuing from the above-identified application.

Dated: 4 March 2003

Signature:

Table 1. The effects of eupomatenoids on inflammatory cell biomarkers

Compound	PGE_2	TXB_2	LTB_2	MPO_2	LDH_2
Eupomatenoid 1	+	+	-	0	+/–
Eupomatenoid 7	+	+	+	+	+
Eupomatenoid 8	+/–	0	+/–	–	–
Licarin A	+/–	+	+	+	+

"+" indicates activity against the target, "+/–" indicates weak anti-inflammatory or cytotoxic activity, "0" indicates no effect, and "-" indicates an inhibitory action.

Peter J Hylands, PhD

Professor Hylands is a pharmaceutical chemist and started his career with academic appointments in the University of Strasbourg as a Royal Society European fellow from 1970-1972 followed by 15 years in the University of London. His special research interest has always been the use of plants in medicine. He joined the commercial world in 1988 as Director of Chemistry at Xenova Limited and then as Research Director of PHYTOpharmaceuticals, Incorporated (USA), both of which were dedicated to the discovery and commercialization of pharmaceuticals derived from natural products. He has acted as a consultant for the United Nations and Commonwealth Science Council. He has worked with European and US-based companies and institutions and is well connected in every continent with regard to natural product research and development. He also serves on a number of boards and advisory boards and as a Visiting Professor in the Department of Pharmacy, King's College London. He cofounded Oxford Natural Products plc in 1998 and serves as the company's Chief Scientific Officer.

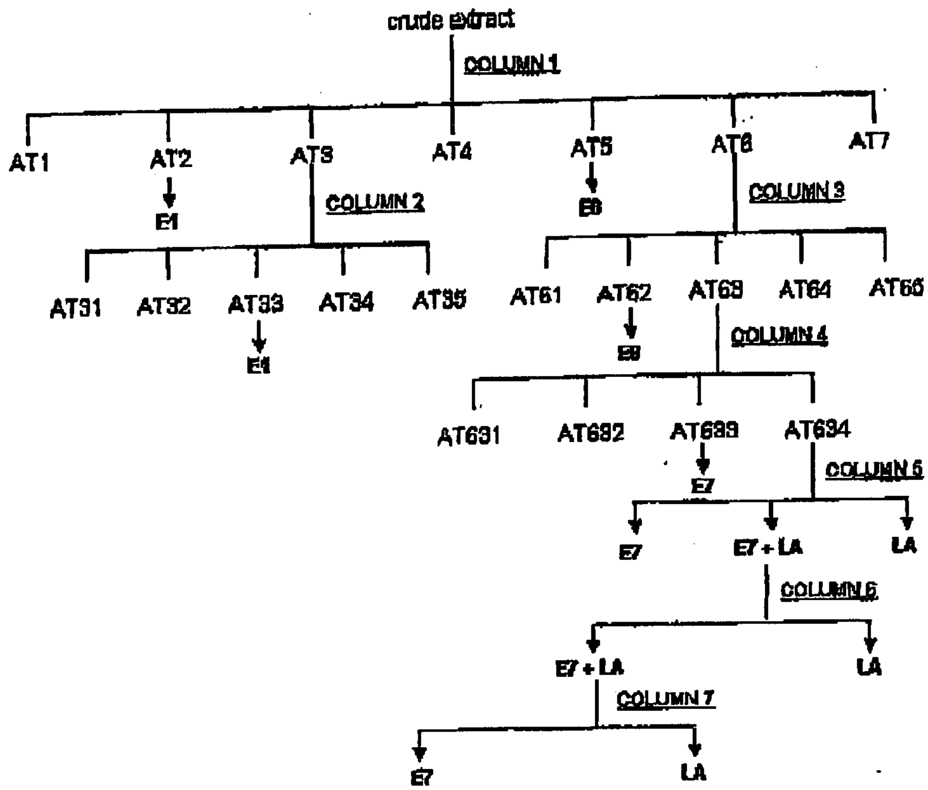
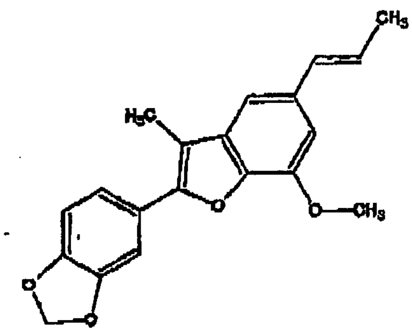
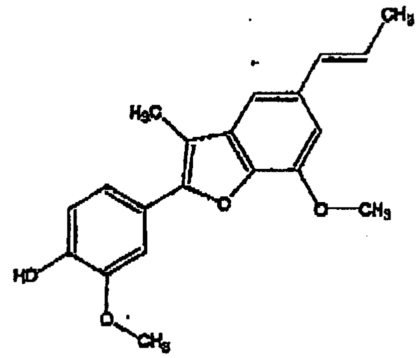


Exhibit B Scheme for the chromatographic isolations of the compounds eupomatenoid-1 (E1), eupomatenoid-7 (E7), eupomatenoid-8 (E8) and licarin A (LA) from the petroleum-ether extract of *A. taliscana* leaf.

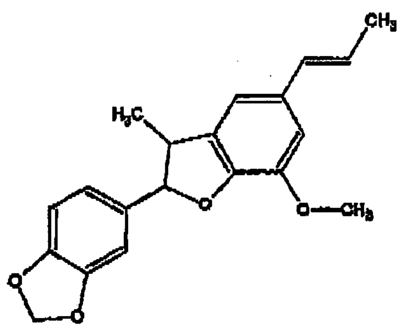
Exhibit C Structures of eupomatenoids from *A. taliscana*



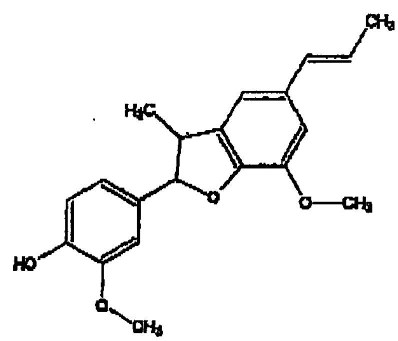
Eupomatenoid-1



Eupomatenoid-7



Eupomatenoid-8



Licarin A

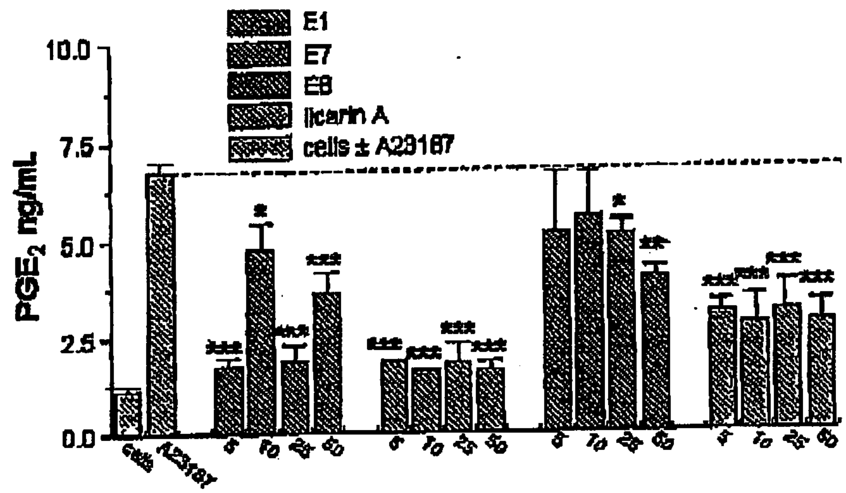


Exhibit D Effect of eupomatenoid compounds at 5, 10, 25 or 50µg/ml on the stimulation by A23187 of PGE_2 formation in rat leukocytes

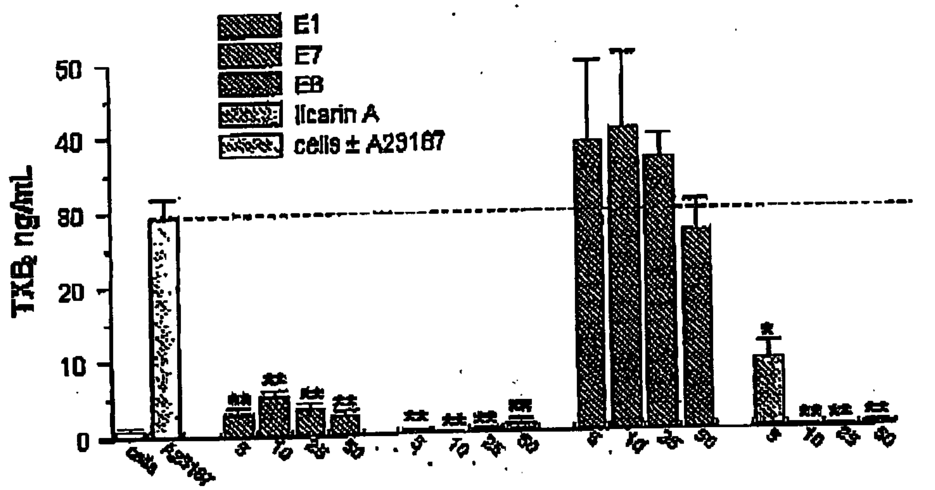


Exhibit E Effect of eupomatenoid compounds at 5, 10, 25 or 50μg/ml on the stimulation by A23187 of TBX_2 formation in rat leukocytes

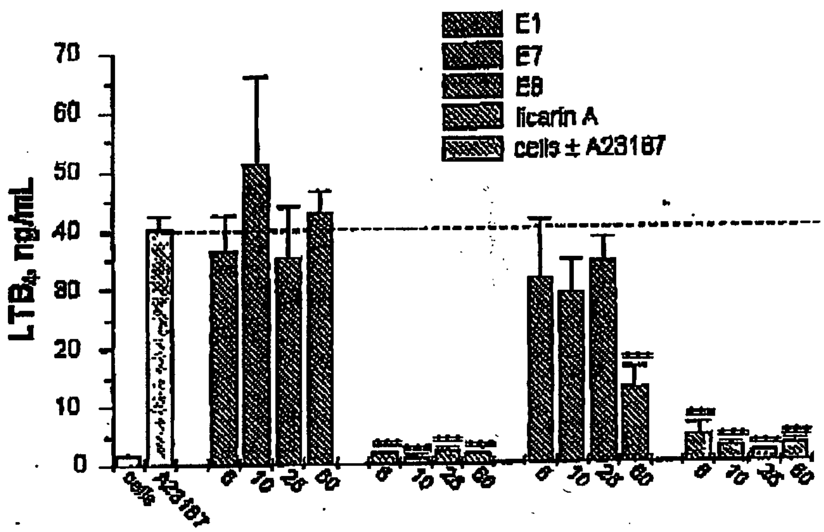


Exhibit F **Effect of eupomatenoid compounds at 5, 10, 25 or 50µg/ml on The stimulation by A23187 of LTB_4 formation in rat leukocytes**

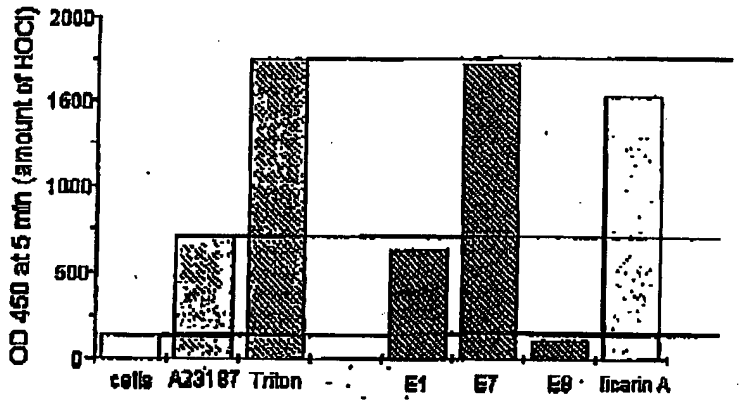


Exhibit G **Effect of eupomatenoid compounds at 50μg/ml on the release of myeloperoxidase from rat leukocytes**

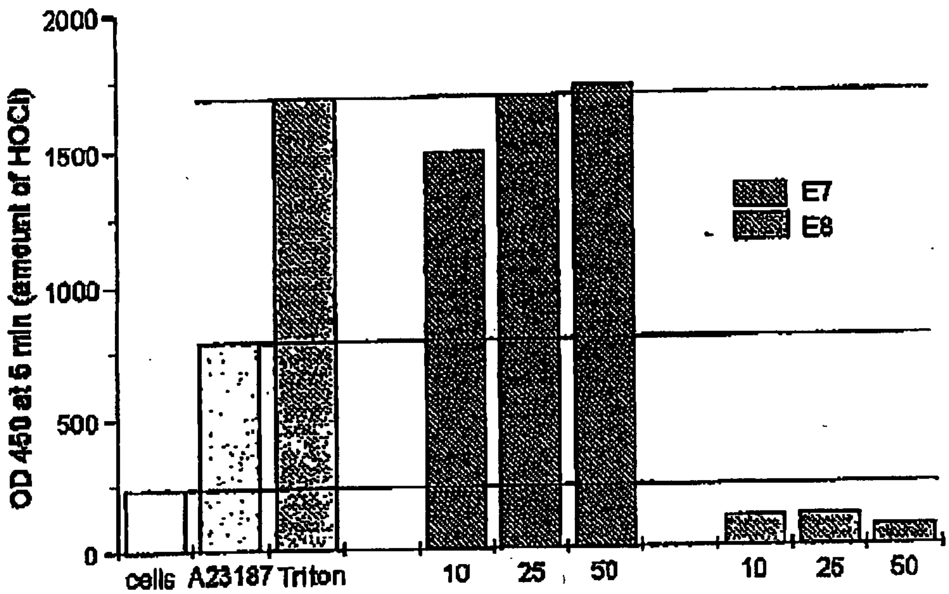


Exhibit H

Stimulation by E7 and inhibition by E8 at 10, 25 or 50µg/ml of release or activity of MPO in rat leukocytes

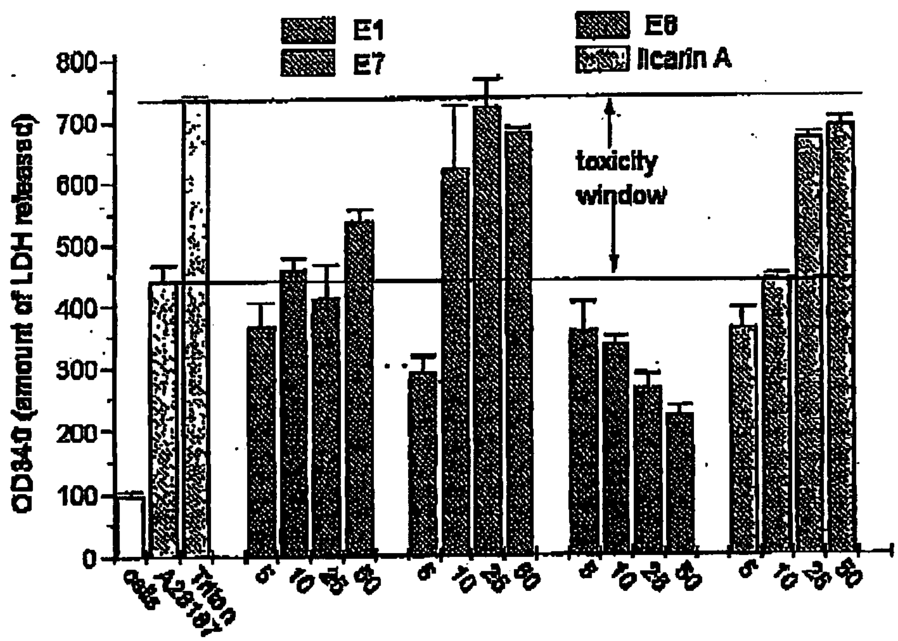


Exhibit I **Effect of eupomatenoid compounds at 5-50μg/ml on release of LDH from rat leukocytes**